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(54) Title: NOVEL GENES OF KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS

(57) Abstract

A human gamma herpesvirus genome known as Kaposi sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8) is present in virtually all AIDS and non-AIDS Kaposi's sarcoma (KS) lesions, as well as in body cavity based lymphomas (BCBL), Multiple myeloma, and in multicentric Castleman's disease. Isolation and DNA sequencing of a 17-kb segment encompassing a HHV-8 divergent locus (DL-B) between ORF11 and ORF17 revealed the presence of nine viral ORFs with gene products related to cellular proteins. These include the complete thymidylate synthase (TS) gene and a dihydrofolate reductase (DHFR) gene, four cytokine genes (vIL6, vMIP-1A, vMIP-1B and BCK) that have not previously been found to be encoded by a virus, and a Bcl2 homologue. This region in HHV-8 also contains the T1.1 abundant lytic cycle nuclear RNA gene and encompasses two genes (or exons) encoding proteins with C4HC3 zinc finger domains of the PHD/LAP subtype. The latter are related to the spliced immediate-early IE1 protein of the bovine gamma-2 class herpesvirus BHV-4 and a similar motif found in HVS ORF12. Transcripts form the IE-1A, IE-1B, DHFR, and MIP-1B genes were all detected by Northern blot hybridization analysis in a BCBL cell line at 12-h after induction with butyrate, but were not present before induction, indicating that these are all primarily lytic cycle genes.

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NOVEL GENES OF KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grants awarded by the National Institutes of Health U01 CA37314, R01 CA73585, and P30 CA06973.

TECHNICAL FIELD OF THE INVENTION

This invention is related to the field of virology, in particular to the area of herpes viruses. More particularly the invention relates to the diagnosis of a herpes virus associated with human diseases.

BACKGROUND OF THE INVENTION

Kaposi's Sarcoma (KS) is the most frequent neoplastic complication seen in patients who are infected with HIV and is especially prevalent in male homosexual AIDS patients (26, 66). It is generally thought to be associated with a sexually and blood borne agent that is transmitted independently of HIV (10). A large proportion of Kaposi's sarcoma (KS) lesions, from both HIV-associated AIDS patients and from non-HIV associated classical and endemic sources, have been found to contain two small DNA fragments representing sequences from a putative novel gamma class herpesvirus genome referred to as KSHV or human herpesvirus-8 (HHV-8) (6, 19, 45). Several additional rare lymphomas, including multicentric Castleman's Disease and Body Cavity Based Large Cell lymphomas (BCBL), but few other

tumor or normal tissue samples contain these same DNA sequences (16-18, 57). Additional recent evidence has shown that these sequences are part of a large episomal viral genome in BCBL cell lines (46, 52), that HHV-8 DNA positive BCBL cell lines can be induced to produce herpesvirus-like particles (52), and that sera from most patients with KS (but not normal sera) have antibodies to HHV-8 proteins (30, 37). In addition, evidence has been found that HHV-8 is also assoicated with Multiple myeloma. (Rettig *et al., Science 276:*1851-1854, 1997.) Together, these results suggest the strong likelihood that this proposed new infectious virus may be the etiological agent of KS (13, 44, 46, 52).

The two small DNA segments found in the original KS lesion studied (19) represent parts of the genes encoding the ORF25 major capsid, the ORF26 minor capsid protein and the ORF75 protein, based on the nomenclature used for the most closely related gamma herpesvirus HVS. The proposed HHV-8 protein fragments of ORF26 and ORF75 display 60% and 30% amino acid identity with their HVS (2, 12) and EHV-2 counterparts (29) and show a slightly more distant relationship to EBV. The known gamma-1 herpesviruses include human EBV and its close relatives in great apes and old world primates (35). The gamma-2 viruses originally included the rhadinoviruses, exemplified by HVS and its close relatives in new world primates, and the cottontail rabbit virus H.sylvilagis. The biological feature of lymphotrophism, including the ability to immortalize and establish a circular plasmid or episomal latent state, was the major common feature that placed them all into the gamma herpesvirus group, and their preferences for B-cells (EBV) or T-cells (HVS) was used as a criterion to discriminate between the gamma-1 and gamma-2 class subgroupings (11, 43, 56). Subsequently, DNA sequence analyses of small segments of EHV-2 and EHV-5 (14, 67), bovine herpesvirus type 4 (BHV-4) and the mouse herpesvirus MHV68, have also revealed closer protein sequence relationships and gene order to HVS than to any other known herpesviruses, despite exhibiting a much broader permissive

host cell range including cultured fibroblasts, and a latency trophism that may include T-cells, B-cells or macrophages (15, 25, 39, 60, 67).

Complete genomic DNA sequence data is now available for three prototype gamma herpesviruses including EBV (7), HVS (3, 4, 48) and EHV-2 (61). EHV-2 appears to represent a distinct subgroup of the gamma-2 herpesviruses based on its overall (G+C)-content of 58%, compared to the much lower 35% and 43% for the unique regions of HVS and BHV-4 (14, 22), together with the presence of large 18-kb terminal direct-repeat structures, rather than the short multi-copy tandem-repeat patterns found at the termini of HVS and BHV-4 (9, 15, 58). Apart from the essentially co-linear organization of major gene blocks (31), each of these gamma herpesviruses contain several clusters of additional genes that are unique to each species and are often not found in any other herpesvirus (32). In EBV, these species-specific or subtype-specific genes include the EBNAs and all other latency-associated genes, the lytic cycle transcriptional and replication control protein ZTA, virus cell-type-specific receptors, and gene products that interfere with or protect against immune surveillance mechanisms (23, 32, 34, 41, 42). All of these optional genes, plus others with as yet unidentified functions, map to within one of six divergent loci in gamma herpesvirus genomes that we refer to from left to right as DL-A to DL-F (39). Amongst the relevant virus-specific and subtype-specific genes that are unique to gamma herpesviruses there is a thymidylate synthase (TS) gene in HVS and EHV-2, but not in EBV or BHV-4 (12, 36, 61). Both EBV and EHV-2, but not HVS or EHV-4, also encode vIL10 genes (54, 61, 65), and amongst the four only HVS encodes a DHFR gene (62). EBV and HVS, but not EHV-2 encode a Bcl-2 homologue and only HVS encodes a vIL17 gene (68). The TS, DHRF, vIL10 and vIL17 genes are highly conserved with the corresponding cellular versions (70 to 85% amino acid identity) and have been suggested to be relatively recent acquisitions as unspliced cDNA versions from the host mammalian genome.

Many intriguing questions arise as to how HHV-8 fits into this classification scheme and what is its evolutionary relationship to the other gamma herpesviruses, as well as whether HHV-8 is indeed an authentic infectious and ubiquitous human virus, rather than a relatively rare pathogen or recent acquisition from some exogenous primate source that has been spread more rapidly within the AIDS epidemic.

Thus there is a need in the art for additional tools for characterizing, diagnosing, and treating HHV-8 which has been found to be associated with a number of human diseases.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an isolated and purified HHV-8 virally encoded protein.

It is another object of the present invention to provide an antibody preparation which is specifically immunoreactive with an HHV-8 virally encoded protein, but is not immunoreactive with a cellular homologue of the protein.

It is an object of the present invention to provide a polynucleotide which encodes one or more of HHV-8 virally encoded proteins.

Another object of the invention is to provide a polynucleotide probe or primer for detecting HHV-8 virus divergent locus DL-B.

Another object of the invention is to provide a polypeptide which comprises at least 13 contiguous amino acids selected from a HHV-8 virally encoded protein.

Another object of the invention is to provide a method for diagnosing an HHV-8 associated disease, such as Kaposi's sarcoma, Castleman's disease, Multiple myeloma, and Body cavity based large cell lymphoma (BCBL).

Another object of the invention is to provide another method for diagnosing an HHV-8 associated disease.

Still another object of the invention is to provide a method of screening test compounds for candidate drugs for treating HHV-8 infections.

These and other objects of the invention are achieved by providing one or more of the embodiments described below. According to one embodiment of the invention an isolated and purified HHV-8 virally encoded protein is provided. The protein is selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, BCK, IE-1A, and IE-1B, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively.

According to yet another embodiment of the invention an antibody preparation is provided which is specifically immunoreactive with an HHV-8 virally encoded protein, but is not immunoreactive with a cellular homologue of the protein. The virally encoded protein is selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, BCK, IE-1A, and IE-1B, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively.

According to still another embodiment of the invention a polynucleotide is provided which encodes one or more of HHV-8 virally encoded proteins. The one or more virally encoded proteins are selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, BCK, IE-1A, and IE-1B, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively. The polynucleotide does not contain HHV-8 genes which are not in the divergent locus DL-B.

In still another embodiment of the invention a polynucleotide probe or primer is provided for detecting HHV-8 virus divergent locus DL-B. The probe or primer comprises at least 12 contiguous nucleotides selected from the group consisting of : SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, and 9.

In yet another embodiment of the invention a polypeptide is provided which comprises at least 13 contiguous amino acids selected from a HHV-8 virally encoded protein. The virally encoded protein is selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, BCK, IE-1A, and IE-1B, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively.

Also provided by the present invention is a method for diagnosing a HHV-8 associated disease, such as Kaposi's sarcoma, Castleman's disease, Multiple myeloma, and Body cavity based large cell lymphoma (BCBL). The method comprises the steps of:

detecting an HHV-8 viral polynucleotide or protein in a body sample of a patient, wherein presence of an HHV-8 polynucleotide or protein indicates an HHV-8 associated disease, wherein the HHV-8 virally encoded protein is selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, BCK, IE-1A, and IE-1B, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively, and the HHV-8 polynucleotide is selected from the group consisting of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, and 9.

According to another embodiment of the invention another method is provided for diagnosing an HHV-8 associated disease. The method comprises:

detecting antibodies in the serum of a patient. The antibodies specifically bind to an HHV-8 protein selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, BCK, IE-1A, and IE-1B, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively.

Another embodiment of the invention is a method of screening test compounds for candidate drugs for treating HHV-8 infections. The method comprises:

contacting a test compound with an HHV-8 virally encoded protein selected from the group consisting of: TS, DHFR, Bcl-2, IL-6, MIP-1A, MIP-1B, and BCK, as shown in SEQ ID NO: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively; and

testing the protein for activity, wherein a test compound which inhibits activity of the protein is a candidate drug for treating HHV-8.

The present invention thus provides the art with a large number of new diagnostic and therapeutic agents for dealing with HHV-8. In addition, it provides the art with targets for development of drugs for treating HHV-8 infections.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Evidence for a Viral Thymidylate Synthetase Gene in Human BCBL DNA as Detected by the Redundant PCR Probe Approach. The diagram shows direct ethidium bromide stained PCR product DNA bands in the appropriate selected size range after fractionation of each set of products by electrophoresis on parallel agarose gels (panels A to E). Size marker lanes containing a multimerized 123-bp ladder series were included in the outside lanes in all gels (not shown). The template samples used in lanes 1 to 10 include control gamma herpesvirus DNA (lanes 1 to 4), BCBL and KS samples (lanes 5, 6 & 9) heavy and light CsCl fractionated C282 DNA samples (lanes 7 and 8), and negative control human placental DNA (lane 10). Each PCR reaction mixture contained 100 ng of template cellular DNA or 3 ng each of the EHV-2 virion DNA, the sucrose gradient fractioned BCBL-R sample and the CsCl fractionated C282H and L samples.

Figure 2: Universal Presence of the HHV-8 TS Gene in Kaposi's Sarcoma DNA Samples as Detected by Direct PCR Analysis. The diagram shows ethidium bromide stained PCR product DNA bands in the appropriate selected size range after fractionation of each set of products on parallel agarose gels (panels A to F). Each reaction mixture received 100 ng of template DNA (except the BCBL-R sample) and 10% of the product DNA was applied to the gel. Samples C282, ASM70, ASM72, 431KAP and AKS1 represent KS lesions (lanes 5 to 9), samples PL/EFFUS, C268 LN, C297 LN, A/SALIVA, and A/SPLEEN represent lymph node biopsy and other control DNAs from AIDS patients (lanes 4 & 11 to 14). UPS75 (panel E) represents a primer pair from upstream of the HHV-8 ORF75 gene from BCBL-R (mapping at a position 1200 to 1500-bp to the right of the Chang 630-bp PCR region). This locus is deleted in some HHV-8 genomes including ASM70 and ASM72 (as well as in HBL6; not shown).

Figure 3: Consistent Patterns of HHV-8, but not EBV Gene Fragments Detected by PCR in Multiple Disseminated KS Lesions from a Single Patient. The diagram shows direct ethidium bromide stained PCR DNA bands in the appropriate selected size range after fractionation of each set of products by electrophoresis through agarose gels (panels A to D). Samples ASM70 to ASM80 represent DNA from multiple KS lesions obtained at autopsy from lung, skin and hilar lymph node sites of a single homosexual AIDS patient in New York in 1984. Samples ASM69 and ASMX were from lymph node biopsies of other AIDS patients with lymphadenopathy in 1984. Samples 431KAP and 431NSL were obtained as skin KS and adjacent normal skin biopsies from a non-HIV associated endemic KS patient from Zaire in 1984.

Figure 4: Relative Map Locations Within the HHV-8 DL-B Region of the TS, DHFR, IE-1A, IE-1B and BCK-Like Genes in Comparison to other Known HHV-8 Genes and the Equivalent Locus in the HVS Genome. The upper portion (A) of the diagram illustrates the arrangement of ORFs in a contiguous 55-kb block at the left-hand end of the gamma-2 class herpesvirus saimiri genome (HVS). Solid bars refer to ORFs that are common to all known gamma herpesviruses, shaded bars indicate ORFs that are found in several gamma 2 viruses and open bars denote ORFs that are unique to HVS. The lower portion of the diagram (C) shows the relative genomic locations of six overlapping phage lambda DNA clones (λD3-80, λVR3a, λVR3b, λC7-1, λ2-1 and λA12-1) containing inserts encompassing a 48-kb segment towards the predicted left-hand end of the HHV-8 genome from the BCBL-R tumor. The sequenced DL-B block totalling 17.4 kb is shown by solid bar. The central portion of the diagram (B) details the relative positions and sizes of all of the currently recognized genes e.g. ORF10, ORF11, vIL-6, DHFR, IE-1B, TS, vMIP-1B, BCK, IE-1A, vMIP-1A, T1.1, Bcl-2 (ORF16), ORF17, ORF18 and ORF19 that map within the region encompassing divergent locus-B (DL-B) of HHV-8. IE-1A and vMIP-1A map in the same orientation as IE-1B and vMIP-

1B, but both lie upstream and several kb to the right on the other side of an unstable (G+C)-rich tandem repeat region [Rpt] within λC7-1. The HVS equivalents of DHFR (ORF2) and TS (ORF70) map towards the extreme left-hand and right-hand ends of HVS, respectively. HVS ORF12 also appears to encode a PHD/LAP class zinc finger protein, and both it and the BHV-4 IE1 gene, which contains two exons with PHD/LAP finger motifs of a similar subclass, map in the same orientation as HHV-8 IE-1A and IE-1B within DL-B of those viruses.

Figure 5: Amino Acid Sequence of the Predicted HHV-8 Thymidylate Synthetase (TS) and Dihydrofolate Reductase (DHFR) Enzymes. (A) Alignment of the TS proteins of HHV-8, H.saimiri (HVS), H.ateles (HVA), equine herpesvirus type 2 (EHV-2), varicella-zoster virus (VZV) and human DNA. Identical regions are boxed. Homology values are listed in Table 1. (B) Alignment of the DHFR proteins of HHV-8, H.saimiri (HVS), human, pig and Drosophila. Identical regions are boxed. Homology values are listed in Table 1.

Figure 6: Predicted Amino Acid Sequences of Zinc Finger and Cys-Bridge Proteins Encoded by HHV-8 DL-B ORFs. Identical regions are boxed. Conserved Cys and His residues characteristic of PHD/LAP zinc fingers and of IL-6 and β-chemokine Cys bridges are indicated by asterisks. (A) Comparative alignment of the IE-1A ORF encoding the 256 amino acid PHD/LAP zinc finger protein and the IE-1B ORF encoding the 333 amino acid PHD/LAP zinc finger protein. Dotted lines denote similarities. (B) Comparative alignments of the HHV-8 ORF-encoding the 114 amino acid β-chemokine-like (BCK) protein with HHV-8 vMIP-1A and vMIP-1B and other β chemokines. The most closely related known proteins to HHV-8 BCK in BLAST and FASTA searches are rat and mouse MCP-1 [S07723, A30209], human T-cell activation protein-2 [A31767] and the human CC-chemokine

[Z75668]. Human MIP1-α and RANTES as well as HHV8, vMIP1A and vMIP1B are also shown for comparison. (C) Comparative alignment of HHV-8 vIL-6 and human IL-6.

Figure 7: Comparison of the Zinc Finger Motifs in HHV-8 IE-1A and IE-1B, BHV-4 IE1 Exons A and B and HVS ORF12 with Other PHD/LAP and RING Finger Subtypes. (A) Alignment of appropriate N-terminal sections of the two putative HHV-8 IE1 ORFs that contain C4HC3 motifs, with those of both exons of BHV-4 IE1 (64) and ORF12 of HVS, as well as Chlamydomonas ZYS-1A, the swine pox virus C7 gene (40), S.cerevisiae SSM4 [P40318] and C.elegans D2089.2, F58E6.1 and M110.2 [Z36948, Z70754 and Z49968]. These eleven motifs represent a distinct subset that we refer to as the BKS versions (BHV4, KSHV & Swinepox) of the C4HC3 PHD/LAP finger motif. The motif designated BHV-4 IE-1B represents nucleotide positions 715 to 873 in Genbank file Accession No. M60043. (B) Examples of two typical standard versions of cellular C4HC3 PHD/LAP zinc finger motifs from the human AF10 and MLLa proteins that are disrupted by translocations in acute myoblastic leukemia (55). (C) Comparative alignment of four prototype C3HC4 pattern RING finger motifs from RAG-1, HSV ICP0, PML and BRCA-1 and a depiction of their predicted cross-braced zinc coordinated structure (28).

Figure 8: Detection of HHV-8 IE-1A, IE-1B and vMIP-1B mRNA Transcripts in a Butyrate Induced BCBL Cell Line. The autoradiographs show the results of Northern blot hybridization analysis of whole cell RNA samples from HBL6 cells using antisense riboprobes generated against the coding regions of HHV-8 IE-1A, IE-1B and vMIP-1B. (A) Induction of both IE-1A and IE-1B mRNA with butyrate (NBA) but not with 5-azacytidine (aza-C). LHS panel. IE-1A riboprobe. RHS panel. IE-B riboprobe. Lane 1, No RNA; 2, uninduced HBL6 RNA; 3, 5-Azacytidine treatment for 24 h; 4, NBA treatment for 24 h. Note that the IE-1A probe showed some cross-reaction with the 28S ribosomal

RNA band in these experiments. (B) Induction of both IE-1A and vMIP-1B mRNA by NBA treatment is blocked in the presence of cycloheximide (CHX). LHS panel, IE-1A riboprobe. RHS panel, vMIP-1B riboprobe. Lane 1, uninduced HBL6 RNA; 2, NBA induction for 4h; 3, NBA induction for 12 h; 4, NBA induction for 12 h plus CHX; 5, Butyrate induction for 24 h plus CHX; 6, NBA induction for 12 h (duplicate RNA sample).

Figure 9: Time Course of Induction of IE-1A and DHFR mRNA in Butyrate Treated BCBL Cell Lines. (A) DHFR riboprobe, HBL6 cells. Lanes 1 to 6, RNA samples prepared at 0, 5, 8, 12, 24 and 48 h after addition of NBA; 7, 12 h after addition of NBA in the presence of CHX. (B) IE-1A riboprobe, HBL6 cells. Lanes 8 to 13, RNA samples prepared at 0, 5, 8, 12, 24 and 48 h after NBA addition. (C) IE-1A riboprobe, EBV negative BCBL-1 cells. Lanes 14 to 17, RNA samples prepared at 0, 5, 12 and 24 h after NBA addition.

Figure 10: Assays for biological activity of HHV-8 encoded vIL-6. (A) Diluted culture medium from REF cells transfected with the vIL-6 expression plasmid pvIL-6, or with a negative control plasmid containing the vIL-6 ORF in the antisense orientation relative to the CMV promoter-enhancer in the pcDNA3 vector (pvIL-6neg), were assayed for IL-6 activity in promoting the growth of B9 cells as described in Materials & Methods. Recombinant human IL-6 (rhIL-6) was used as a positive control (data not shown) and to enable the calculation of the activity (U/ml) of vIL-6. Transfected REF cell medium containing vIL-6 was harvested either at 2 and 4 days posttransfection (experiment 1, left) or at 3 days posttransfection (experiment 2, right). For each supernatant dilution used, the assays were performed in quadruplicate. (B) Hep3B cells were grown in 25cm^2 flasks and transfected with 10μg of pSG5 (empty vector), pSVvIL-6 (vIL-6 expression vector), pvIL-6, or pvIL-6neg. Other Hep3B cells were either untreated ("medium") or treated with 500U/ml rhIL-6 (Gibco-BRL). After 48h, the cells were harvested, 5μg total cell RNA per sample was size fractionated for northern blot analysis, and

membranes were hybridized with ^{32}P-radiolabelled α_1-acid glycoprotein (AGP) probe DNA sequences. The positions of 28S and 18S ribosomal markers and the estimated size of the detected AGP transcripts are indicated.

DETAILED DESCRIPTION

It is a discovery of the inventors that the DL-B locus of HHV-8 DNA from both KS and BCBL samples encodes as many as nine ORFs that are related to known cellular proteins, including TS and DHFR enzymes and two LAP zinc-finger motif proteins that are characteristic of several other gamma-2 class herpesviruses. These HHV-8 encoded cytokine genes, (Il-6, MIP-1A, nd MIP-1B) and a Bcl2 homologue also mapped to this locus. In addition, a fourth HHV-8 cytokine gene related to β chemokines and referred to as BCK has been found.

Isolated and purified proteins from the DL-B locus are provided. These proteins can be made using the nucleotide sequences which encode them naturally in the virus. They can also be made using other variant coding sequences, or they can be made synthetically. Isolated and purified proteins have been removed from the cellular millieu, and have been purified to at least form 10% of the proteins (by weight) in the preparation. Preferably the preparation will be more pure, so that the HHV-8 protein forms at least 25, 40, 50 60, 75, 85, 90, or 95% of the protein in the preparation. Any means known in the art can be used to purify and isolate the protein from cells. Particularly useful methods are based on the sequence information dislcosed here, such as immunological methods. Such methods rely on the use of antibodies which bind to the protein with specificity.

Polypeptide portions of the proteins can also be useful, both as immunogens and as antigens or reagents. These can be preferably at least 13, 15, 17, 19, 21, 23, 25, 30, 35, or 50 contiguous amino acids of the respective proteins. The polypeptides can also be covalently linked to other amino acids,

as in fusion proteins which link the viral sequences to other useful sequences, such as enzymes, secretion leader sequences, and immunological tags.

Antibodies can be made according to any method known in the art. Whole proteins, polypeptide portions of proteins, fusion proteins, can all be used to immunize animals to raise an humoral immune response. The antibodies can be purified, for example, using affinity chromatography. The antibodies can be monoclonal, if the spleen of the immunized animal is used to make hybridomas or otherwise immortalized cells. Specifically binding antibodies are those which bind with a greater binding affinity to the particular HHV-8 protein than to other proteins. Preferably the greater binding affinity is at least two-fold and preferably at least ten-fold greater than to other proteins.

Polynucleotides according to the invention are all or portions of genes which have been purified away from other non-DL-B HHV-8 sequences. Preferably other cellular polynucleotides have also been removed. The polynucleotides can be in expression constructions, labeled to form a probe, appended with a restriction enzyme site, or in any other form which may be useful for a particular purpose. They may also be used as primers for synthesis and amplification. In addition, they can be joined to sequences to form ribozymes, as is well known in the art. Preferably at least 12, 20, 30, 40, 50, 100, or 200 contiguous nucleotides of the natural HHV-8 sequence are used. Means for synthesizing, isolating, labeling, and cloning polynucleotide sequences are well known in the art. The polynucleotides can be cDNA or viral genomic DNA; they may also be put in constructs in inverse orientation to form anti-sense constructs which express RNA complementary to the native mRNA. They may also be part of ribozymes.

The viral promoters and regulatory elements may be used to control other (non-viral) sequences in chimeric constructs. Conversely, non-viral promoters and regulatory elements can be used to control the viral sequences disclosed herein.

Methods for diagnosing a disease associated with HHV-8 include any by which HHV-8 viral polynucleotides or proteins are detected in a body sample of a patient. The body sample may be a tissue sample, blood, serum, sputum, saliva, etc. Any source of viral proteins or nucleic acids will provide a suitable sample. It may be desirable to treat the patient or the sample prior to the step of testing with a drug or agent which induces viral expression. Such drugs or agents include butyrate, phenylbutyrate, and azacytidine, and derivatives thereof. Detection methods include any that are known in the art. Protein and antibody detection methods include without limitation: ELISAs, immunoblots, immunoprecipitation. Autoantibodies can be detected using the same techniques. Nucleic acid detection methods include PCR, Northern blots, Southern blots, in situ hybridizations, etc. Any convenient technique or format can be employed to test for the disclosed HHV-8 proteins and gene sequences. The presence of HHV-8 DL-B proteins or nucleic acid sequences in the sample indicates an HHV-8 infection which may develop into one of the diseases which HHV-8 plays an etiological role. Antiviral treatments may ameliorate or alleviate the development of the full disease symptoms.

The HHV-8 proteins of the present invention can be used to screen for test compounds which are useful for treating HHV-8 infections. Such screening methods typically employ the contacting of a test compound with one of the virally encoded proteins and determining whether the activity of the protein is inhibited by the test compound. A test compound which inhibits function of one or more of the viral proteins is a candidate drug for treating HHV-8. Further testing to determine that the test compound does not inhibit the cellular homolog of the HHV-8 protein will establish specificity for the virus. These screening assays can be performed using *in vitro* or *in vivo* formats. In the former, isolated proteins are contacted in a cell-free system with the test compounds. In the latter cells which express the viral proteins are contacted with the test compounds. The functional assays for the protein can be performed thereafter, either *in vitro* or *in vivo*. As described herein, the

viral proteins of DL-B share activities with their cellular homologues, therefore similar assays can be used for the viral proteins as for the cellular homologues.

The proteins and polypeptides of HHV-8 DL-B can be expressed according to any method known in the art. They can be expressed in bacteria, using bacterial expression cloning systems. They can be expressed in *in vitro* translation systems using HHV-8 mRNA as a template, or in coupled transcription/translation systems, using HHV-8 DNA as a template. The DL-B genes can also be expressed in mammalian cells, either in transiently transfected cells or in stable transfectants, such as in CHO cells. Methods for such expression are known in the art. Using such expression systems it has been found that the viral DHFR confers methotrexate sensitivity on mammalian cells, and the thymidylate synthase confers 5-fluorouracil senstivity on *E. coli* cells. Moreover, using such expression systems it has been demonstrated that the viral homologues of cellular cytokine proteins have similar or overlapping biological function as the cellular proteins.

The thymidylate synthetase (TS)-like gene fragment amplified from the BCBL DNA sample was found to encode part of a protein that had 70% amino acid identity to both human TS and to the ORF70 proteins of HVS and EHV-2 and was slightly further diverged from the varicella zoster virus (VZV) version. Since EBV does not possess a TS gene and the mammalian versions are spliced, we interpreted that this probably represented an HHV-8 gene. This conclusion was confirmed by the isolation of a phage lambda clone from a BCBL genomic DNA library that also contained diverged homologues of other gamma herpesvirus genes such as ORF6 and ORF10. The HHV-8 TS fragment was found to be present consistently in all samples of KS or BCBL DNA tested from twelve different patients, but was not found in any non-KS or BCBL control samples. Based on this locus as a probe, a set of overlapping phage lambda clones were identified from the BCBL-R library that linked up into a contiguous 48-kb segment, encompassing the equivalent of HVS ORF6 to 31 and overlapped with the left-hand end of the previously described 20-kb

ORF20 to ORF35 segment of KS HHV-8 (46). We focused our attention only on a region surrounding the TS gene of HHV-8 with the anticipation that it would represent the equivalent of the gamma herpesvirus divergent locus DL-B, which we hoped would reveal additional clues about the evolutionary relationship of HHV-8 to other gamma herpesviruses.

Combined physical mapping and DNA sequence analysis has revealed that HHV-8 contains an expanded region of nearly 18-kb of DNA within the DL-B region between homologues of HVS ORF9 and ORF17, compared to only 7-kb in this region in HVS (Fig 4). Furthermore, HHV-8 encodes at least nine genes whose products have homology to cellular proteins or motifs within this region, including ORFs representing the TS, DHFR, BCK-like, and two PHD/LAP zinc-finger class proteins described here, as well as vIL6 and two vMIP-1 homologues (50) and a highly diverged but positionally conserved version of HVS Bcl-2 (21). At least five of these genes produced distinct lytic cycle transcripts at 12 h after butyrate induction in both EBV positive and EBV negative BCBL cell lines that carry apparently latent HHV-8 plasmid genomes. Note that only Bcl-2 and one PHD/LAP zinc finger protein are also found at this equivalent location in the prototype gamma-2 herpesvirus genome of HVS, together with vIL17 (ORF13), the IE-G (ORF14) protein and a CD59 homologue (ORF15); (4). However, HVS and EHV-2 both contain homologues of the TS gene (referred to as ORF70) near the right-hand-end of the unique segments of their genomes. Furthermore, only HVS also contains a DHFR gene, although this is designated ORF2 and lies near the extreme left-hand end of the genome. None of the other eight genes are present in the EHV-2 genome.

Based on their very different genomic locations, and the fact that the HHV-8 version of the DHFR protein is much farther diverged from the HVS version than the HVS version is from human DHFR (48% identity compared to 84% identity), we conclude that the two gamma herpesvirus DHFR genes were probably acquired from their hosts as separate events at different time

periods in gamma herpesvirus evolution. Assuming that the two DHFR genes did not evolve at different rates, capture by the HHV-8 progenitor must have occurred long before that in a common progenitor of HVS and EHV-2. The TS genes may also have had different evolutionary origins, although based on their equivalent degrees of homology and divergence from the human version (66% for HHV-8 and 69% for HVS) we cannot exclude the possibility of a secondary translocation in this case. Varicella-zoster virus (VZV) is unique amongst alpha herpesviruses in also containing a TS gene (63% identity), which again is positioned at a different genomic location than are either of the two gamma virus TS loci. Furthermore, there is also the precedent from the Bcl-2 homologues in EBV and HVS, and the vIL10 homologues in EBV and EHV-2, which also both represent acquired cellular cDNA genes that occupy different positions in their respective genomes. The apparent duplication of vMIP-1 and IE1 genes and the acquisition of three distinct types of cytokines within the same divergent locus by HHV-8, but the presence of vIL17 instead at this locus in HVS, also attests to the relative frequency of these recombinational events and the high genomic fluidity at this site.

Finally another gamma herpesvirus, BHV-4, possesses apparent evolutionary homologues of the two HHV-8 zinc-finger proteins described here, but neither EBV or EHV-2 do so. In this case, the BHV-4 IE1 gene maps at the equivalent location and in the same orientation as the HHV-8 genes (i.e. leftwards within DL-B). We have also identified HVS ORF12 as another positionally equivalent member of this family. The two HHV-8 versions are much more closely related to each other than to those in the other two viruses (Fig 6A) and, although direct homology between the HHV-8, BHV-4 and HVS versions of these proteins does not extend beyond the N-terminal zinc-binding motifs, they all resemble each other to the extent of having several internal hydrophobic domains. Only one of the BHV-4 motifs was recognized originally (64) and it was suggested then to be closely related to the zinc finger motif in the HSV ICP0 (or IE110) transactivator protein. However, our

analysis has revealed that within the single IE1 transcription unit of BHV-4 both exons 4 and 5 contain a C_4HC_3 type of zinc-finger motif, which is distinct from the C_3HC_4 motif in ICP0. In fact, the gamma-2 herpesvirus motifs all closely resemble a subset of C_4HC_3 zinc finger motifs within the PHD/LAP class found only in the C7 protein encoded by swine pox virus (40) or in the ZYS1A/B proteins of Chlamydomonas and in several S. cerevisiae and C. elegans proteins, but otherwise they differ significantly from most other C_4HC_3 PHD/LAP class motifs, and from the better known C_3HC_4 and C_2HC_5 classes of RING and LIM zinc-finger motifs (Fig 7). The zinc-binding motif in the HSV ICP0 nuclear regulatory protein and its alpha herpesvirus relatives is of the RING-finger class, and there is no obvious resemblance otherwise to the gamma-2 herpesvirus IE1 zinc finger proteins, which appear likely to be membrane proteins.

The overall similarity amongst the two PHD/LAP zinc-finger containing proteins in HHV-8, the two IE1 exons in BHV-4 and ORF12 in HVS, is sufficient to conclude that these proteins in the three viruses have a common evolutionary origin and presumably are likely to have related functions. Therefore, we have provisionally designated the HHV-8 versions as IE-1A and IE-1B based on previous evidence that at least one of the BHV-4 versions (exon-4) is expressed as a classical lytic cycle IE class mRNA after infection of permissive cells with BHV-4 virions (64). The ability of CHX to block HHV-8 IE-1A and IE-1B transcription after butyrate induction in BCBL cell lines is not necessarily relevant to the question of IE characteristics after primary infection. The RING class zinc finger domains in HSV ICP0 are known to have an unusual cross-braced zinc coordination pattern creating distinctive alpha helical and β sheet structures that are believed to be protein:protein interaction domains (27, 28); however, the PHD/LAP motif is structurally sufficiently different that no predictions about function can yet be made. Interestingly, at least three human members (AF10, MLLT6 and MLLa)

of the leukemia associated protein domain (LAP) family with similar C_4HC_3 zinc finger motifs are disrupted in acute myeloid leukemias (55).

We conclude that gamma herpesviruses are likely to have both captured and lost cellular cDNA genes such as Bcl-2, IL10, TS and DHFR on multiple occasions during their evolution, and that in similarly acquiring both multiple cytokine and cytokine receptor genes etc, they have undergone rather dynamic additions and subtractions to their genome content, especially within the divergent loci. Presumably, separate independent acquisitions of the same gene may reflect some selective advantage to encoding these proteins. The whole process of gaining, losing or rearranging genes at divergent region recombination "hot spots" may be a relatively modern reflection of the ongoing long-term evolutionary events that also generated the more stable subclass-specific segments of alpha, beta and gamma herpesvirus genomes seen now, and perhaps even those events that originally incorporated the common replication enzyme genes of all of these viruses.

With regard to the overall relationship of HHV-8 to other gamma herpesviruses, the amino acid comparisons of the stable conserved MCP proteins presented by Moore et al (46) clearly support a slightly closer evolutionary history to HVS than to EHV-2 and much further divergence from EBV. The patterns of gene conservation within DL-B and DL-E also reveal more similarity between HHV-8 and HVS than to EHV-2 with regard to the presence of the conserved ORF10, ORF11, ORF12(IE1), ORF16 (Bcl-2), and ORF72 (cyclin-D) (20) genes. However, HVS and EHV-2 are more similar to each other than to HHV-8 in possessing a common ORF70 TS gene locus, whereas all three of them possess an ORF74(GCR) gene (33). Furthermore, HHV-8 appears more similar to BHV-4 than to either of the others with regard to the conservation of two IE1 zinc finger motif proteins or exons, but BHV-4 apparently lacks any DHFR, TS, cyclin-D, GCR, IL10, Bcl-2 or cytokine genes (39). Obviously, the potential of the HHV-8 encoded TS and DHFR proteins as novel targets for antiviral therapy, the expectation that at least one of the

IE1-like zinc-finger protein(s) may serve as an IE class lytic cycle regulator for this virus, and the likely biologically intriguing consequences of expressing viral encoded inflammatory cytokines will lead to further more extensive studies of the function and expression of these genes.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Use of Redundant PCR Primers to Detect Gamma Herpesvirus Genes:

Pairs of redundant PCR primers encoding short well-conserved amino acid blocks common to known gamma herpesvirus versions of the uracil DNA glycosylase (UDG), thymidylate synthase (TS), interleukin-ten (IL10) and dihydrofolate reductase (DHFR) genes were synthesized and tested for amplification of DNA products at low stringency on six different species of gamma herpesvirus DNA preparations. These samples included human EBV (present in the Jijoye or Raji B-lymphoblast cell lines), baboon and orangutang gamma-1 herpesviruses (H.papio and H.pongo) in B-lymphoblast cell lines, squirrel monkey gamma-2 (HVS) DNA from infected Vero cells, purified equine gamma-2 (EHV-2) virion DNA and mouse gamma-2-like (MHV68) virion DNA. Note that the spliced cellular versions of these genes are not expected to be detected because of their larger size, whereas the introns are missing in the viral versions. The IL10 PCR primers proved to detect a vIL10 gene in EBV, H.pongo, H.papio and EHV-2, but not in any of other samples (not shown), whereas the UDG probes detected viral UDG genes in all six gamma herpesvirus DNA samples tested (not shown). The DHFR primers, which were designed based on blocks of homology between the HVS version and human and mouse DHFR, failed to detect a DHFR gene in any sample other than the HVS DNA (Fig 1B, lane 3). As expected, the TS primers detected TS genes in both EHV-2 and HVS (Fig 1C, lanes 2 & 3), but not in

EBV, MHV68 or the two non-human primate gamma-1 DNAs (Fig 1B, lanes 1 & 4).

Detection of a Viral TS Gene in BCBL and KS DNA by the Redundant PCR Approach:

Amongst the four redundant gamma class PCR primers described above only one, the TS pair, proved to detect an apparent HHV-8 homologue. In the initial studies, three potential HHV-8 positive DNA samples were tested for direct ethidium bromide stained PCR products, including an AIDS-associated body cavity based lymphoma tumor (BCBL-R), a biopsied KS lesion (AKS1) and KS autopsy sample (C282). Analysis of these DNA samples for direct ethidium bromide stained PCR products with a sensitive HHV-8 ORF75 primer pair revealed considerably more HHV-8 DNA in the BCBL-R sample (Fig 1A, lane 5) than in either of the two KS lesions (Fig 1A, lanes 6 & 9). This primer pair proved to be highly specific for HHV-8 and failed to detect homologous sequences in MHV68, EHV-2, HVS, Jijoye (EBV) or human placental DNA samples (Fig 1A, lanes 1 to 4 & 10). However, the BCBL-R and C282 samples were also strongly positive for EBV and the AKS1 sample was weakly positive for EBV as detected with an EBNA-1 specific PCR primer pair (Fig 1E, lanes 5, 6 & 9). As expected, both EBV and HHV-8 DNAs were enriched in the C282 KS sample by banding in a CsCl density gradient to partially separate (G+C) rich viral DNA (Fig 1A and E, lane 7; C282H) from the cellular DNA fraction of lower G+C content (lane 8, C282L).

The vIL10, UDG and TS primers were also all positive with the BCBL-R and C282 KS samples, but not surprisingly, sequencing of the products revealed that the vIL10 and UDG products obtained were derived from a subtype A EBV genome. However, EBV lacks a TS gene and the sequence of the TS PCR product obtained from the BCBL-R sample (Fig 1C, lane 5) revealed the presence of a novel fragment of a TS gene closely related to that in HVS. The 300-bp PCR product contained a partial ORF with 67% identity

to HVS and H.ateles TS and 63% to the human version. This TS fragment was not detectable directly with the redundant primer in either the AKS1 or C282 KS DNA samples (Fig 1C, lanes 6 & 9), but it was detected within the high (G+C) content fraction in the viral DNA enriched sample after banding in CsCl (C282H; lane 7). Furthermore, the DNA sequence of the C282 KS TS fragment proved to be identical to that from the BCBL-R fragment. Based on these sequences, a new HHV-8 vTS specific primer pair was designed, which now detected a 280-bp TS gene fragment directly in all three of the HHV-8 positive samples (Fig 1D, lanes 5, 6 & 9) as well as in EHV-2 and HVS (at low stringency, 48°), but not in MHV68 or Jijoye EBV, nor in the human placental DNA control (Fig 1D, lanes 1, 4 & 10).

Sources of KS and BCBL DNA and Gammaherpesvirus DNA Samples:

A variety of discarded KS-related autopsy and biopsy samples were accumulated from both AIDS and non-AIDS patients at Memorial Hospital Sloan-Kettering Institute (New York) and from Zaire in 1983 and 1984 (5), as well as a variety of additional specimens from Johns Hopkins Hospital and the National Institutes of Health in 1995. These included AIDS KS autopsy samples labelled C282 and ASM70 to 80 (New York, 1984), endemic African KS DNA samples 431KAP and 431NSC (from Zaire, 1984), AIDS-associated female KS DNA samples ST1, ST2 and ST3 (from Uganda, 1995), fresh frozen AIDS associated AKS1 and non-AIDS associated EKS1 lesion samples (from Baltimore, 1995), an AIDS KS lesion DNA sample referred to as AKS2 (from NIH, 1995) and a paraffin-embedded AIDS-associated KS lesion AKS4 (from Baltimore, 1995). Other HHV-8 positive samples included the original BCBL tumor DNA (BCBL-R) used to prepare the phage lambda library (from NIH, 1995) and DNA from the HHV-8 plus EBV positive HBL6 cell line derived from the BC1 cell line (16). DNA samples from all tumor, biopsy and tissue or cell culture sources were extracted with pronase and phenol followed

by ethanol precipitation and dialysis against Chelex. DNA concentrations were measured by UV absorption.

Total cell DNA from cultured human Raji and Jijoye B-lymphoblasts carrying latent state EBV genomes was prepared by pronase and phenol extraction followed by ethanol precipitation. Similar procedures were used to extract DNA from HVS lytically-infected Vero cells, MHV68 infected MEF cells and both the H. papio infected baboon B-lymphoblast cell line (BA34) and a newly isolated H.pongo infected orangutang B-lymphoblast cell line. Purified EHV-2 virion DNA was obtained from Dennis J. O'Callaghan (Univ. of Louisiana, Shreveport).

Redundant Primers and PCR Analysis:

The redundant PCR primers originally used to detect a 300-bp gamma 2 TS band were 5'-GGRCCYGTSTAYGGRTTYCAGTGG-3' (LGH1967) and 5'-CTGGC(Y/G)ATGTTGAARGGRAC(Y/G)CC-3' (LGH1968). The second generation HHV-8 specific TS PCR primers used to detect a 280-bp band were 5'-GGGTTCCAGTGGAGGCAC-3' (LGH2018) and 5'-ACGTGGATCCCTCTGACAACC-3' (LGH2019). The HHV-8 ORF26 PCR primers used to obtain 330-bp bands, namely 5'-GGATGGATCCCTCTGACAACC-3' (LGH1701) and 5'-ACGTGGATCCGTGTTGTCTACG-3' (LGH1702), were based on the sequence of the KS330Bam fragment of Chang et al (19). The ORF75 PCR primers used to obtain 530 bp amplified bands, namely 5'-CTAGAGATCTGTTTAGTCCGGAG-3' (LGH1984) and 5'-GTACGGATCCACGGAGCATAC-3' (LGH1704) , were derived from the KS631Bam DNA sequence of Chang et al (19). The UPS75 PCR primers used were 5'-CAGTTACATGTAGCCATG-3' (LGH2024) and 5'-CTGCATCAGTACACTATTC-3' (LGH2025), which were derived from DNA sequence analysis at the left-hand end of a BCBL-R phage lambda DNA clone (λB5-1) identified by hybridization with the KS631Bam ORF75 probe. The

EBNA-1 primer pair used to obtain 410-bp bands were as follows: 5'-TGTAGGGGAAGCCGAT-3' (LGH606) and 5'-CAATGGTGTAAGACGAGATTG-3' (LGH242).

PCR amplification was carried out with Taq DNA polymerase (Boerhinger-Mannheim) at 94° from 1 min, 48° or 54° for 1 min and 72° for 2 min over 35 cycles in a Techne-PHC3 Thermal Cycler. In most cases with cellular DNA 100 μg of template was used and 10% of the product was analysed by electrophoresis on 2.25% Nu-Sieve agarose gels (FMC, Rockland, Maine) followed by direct ethidium bromide staining and UV-photography. Nested PCR procedures were avoided to reduce the potential for contamination complications. For PCR sequencing, the size selected DNA product was recovered from the gel slice in 2.5M NaCl in TE by melting at 65° for 5 min, followed by phenol/chloroform phase extraction and precipitation with 40% isopropanol, 0.4M $NaClO_4$ and 40μg/ml glycogen at 20°C, washing the pellet with 70% ethanol, drying and dissolving in 1x TE. The double-stranded DNA cycle sequencing system (GIBCO-BRL Life Technologies) was employed with [^{32}P] γ ATP-labeled primers using T4 polynucleotide kinase and analysis on 6% polyacrylamide/7M Urea gels and autoradiography.

EXAMPLE 2

Phage Lambda Library Preparation:

An EMBL3A phage lambda genomic DNA library was generated directly from a Sau3A partially digested preparation of cellular DNA from an EBV plus HHV-8 positive BCBL-R tumor DNA sample after size fractionation into 15 to 20-kb segments by sedimentation in a sucrose density gradient. A second genomic library in the lambda DASHII vector was also generated from the same BCBL-R DNA sample. Two initial clones, referred to as λA12-1 and λD3-80 were detected and isolated after multiple rounds of plaque lift blot

hybridization with [^{32}P]-labeled DNA products representing the ORF26 KS330Bam fragments described by Chang et al (19) or from a plasmid containing the TS300 PCR fragment described in the text. Four additional overlapping clones, λC2-1, λC7-1, λVR3a and λVR4a, were then selected by chromosome walking procedures. The physical maps and orientations of these clones, which contain inserts of approximately 18-kb for the λEMBL3 versions, and 12-kb for the λDASH versions were established by restriction enzyme digestion patterns and direct double-stranded primer-based DNA sequencing of the insert termini.

Subcloning and DNA Sequence Analysis:

Three plasmid subclones containing adjacent 1.2kb XhoI to EcoRI, 2.5kb EcoRI to Eco-RI and 2.4kb EcoRI to EcoRI fragments surrounding the TS gene locus from phage λD3-80 DNA and an adjacent 2.5kb EcoRI to BamHI fragment plus a 6.7 kb BamHI to BamHI fragment encompassing the Bcl-2 homologue from phage λC7-1 DNA were subcloned in Bluescript plasmids and then randomly sheared and inserted into M13 for standard shotgun manual dideoxynucleotide sequencing analysis as described previously (47, 49). Extensions of each sequence block inwards towards a high (G+C) content heterogenous region between the BCK and IE-1B genes was carried out by direct cycle sequencing and primer walking procedures on λD3-80 and λC7-1 DNA.

DNA and protein sequence data for the HHV-8 BCBL-R genes encoding vIL-6 (U67774), DHFR (U83347), IE-1B (U83350), TS (U83348), vMIP-1B (U67775), BCK (U83351), IE-1A (U83349), vMIP-1A (U74585) and Bcl-2 (U67773) have been filed with Genbank and are available under the listed accession numbers.

EXAMPLE 3

Detection of a Viral TS Gene in All HHV-8 Positive KS Specimens:

As a further test of the validity of our TS fragment as an authentic HHV-8 gene, we examined 100ng samples of three additional known HHV-8 positive KS DNA specimens (ASM 70, ASM72, 431KAP) and a selection of negative control DNA specimens, including two lymph node biopsies from HIV positive patients with lymphadenopathy, plus human pleural effusion cell, saliva and spleen DNA from AIDS patients. Even when used at higher stringency (54° compared to the earlier 48°), which reduced the cross-over signal from HVS DNA, the HHV-8 specific TS primers (Fig 2C) produced exactly matching 280-bp band patterns to those of both the HHV-8 ORF26 and ORF75 primers (Fig 2A and B) for all six positive and eight negative DNA samples tested. In comparison, the EBV EBNA-1 primers were strongly positive with Jijoye EBV, BCBL-R, C282 and one non-KS control sample and weakly positive with ASM70, whereas the other three KS samples (ASM72, 431KAP and AKS1) were negative for EBV at this level of sensitivity (Fig 2E). All of the negative samples produced amplified globin PCR products (Fig 2F).

In a second comparison of this type, we screened a collection of autopsy samples from seven distinct KS lesion sites (including skin, lung and hilar lymph node) from a 1984 New York homosexual AIDS patient with aggressive disseminated disease (ASM 70 to 80). Again, all three HHV-8 PCR primers (ORF26, ORF75 and vTS) produced virtually identical patterns of positive signals in ten DNA samples, and negative results in four samples (Fig 3A, B & C). Amongst the seven KS lesions from the same ASM patient, four were positive for EBV EBNA-1, one strongly so (Fig 3D, lane 8, ASM79) and three were negative. In addition, two more lymphadenopathy biopsy samples from patients later recognized to be in early stages of AIDS, were strongly positive for EBV, but were negative for HHV-8 (Fig 3, lanes 2 & 10, ASM69, ASMX). The two other HHV-8 positive samples shown came from a Zaire non-HIV associated endemic KS lesion biopsy (431KAP), and an adjacent non-KS skin sample (431NSC) from the same patient, which were also both EBV negative (Fig 3, lanes 11 and 12).

Overall, we have similarly detected the putative HHV-8 TS DNA fragment by direct PCR amplification in BCBL or KS DNA samples from 12/12 different patients and 22/22 distinct lesions in samples that were also positive for HHV-8 ORF26 and ORF75, but never in DNA samples that were negative for HHV-8 with the other two primer pairs. Furthermore, a second primer pair based on the original BCBL-R TS sequence gave the same results and the latter 280 bp TS PCR products also all proved to be identical by DNA sequencing in KS or BCBL samples from six different patients (not shown).

The ASM70 and ASM72 KS samples proved to be unique in that in contrast to the other KS samples tested they were both negative for amplification of the UPS75 primer pair (Fig 2D, lanes 6 & 7), which represents a region from -860 to -210 upstream from the first ATG in the HHV-8 ORF75 coding region. We have found this region to be deleted in a subset of HHV-8 genomes, which we refer to as the C subgroup, that includes the ASM70 KS sample and the HBL6 cell line. A comparison of strain variability amongst the DNA sequences over a total of 2500-bp within the ORF26, ORF75 and UPS75 regions of each of the twelve distinct HHV-8 genomes described here is presented in the report by Zong et al (70).

EXAMPLE 4

Isolation and Sequence of Complete Genes for HHV-8 TS and DHFR from a BCBL-R Genomic Phage Lambda Library:

A phage lambda EMBL library of size-selected partial Sau3A cleavage fragments from the same BCBL-R DNA sample described above was generated and screened for the presence of clones that hybridized with a labelled probe DNA representing a plasmid cloned sample of the original 270-bp TS PCR product. The first positive clone isolated (λD3-80) proved to contain a DNA sequence at the left-hand end that showed considerable homology to the ORF6 single-stranded DNA-binding protein genes of HVS

(60% amino acid identity over positions 749 to 808 in the HVS version) and less to EHV-2 and EBV (47% and 48% amino acid identity over the same region). The 18-kb insert of this clone overlaps on the right-hand side with the left-hand end of another lambda clone (λC7-1) which also hybridized with the TS probe. This second phage clone contains a homologue of the HVS ORF22 (glycoprotein H) gene at its right-hand end and was originally selected because of overlap with two more lambda clones (λC2-1 and λA12-1) extending further to the right covering the region between ORF20 and ORF31. Overall, we generated a set of six overlapping λ clones containing a contiguous 48-kb section of the HHV-8 genome extending from ORF6 to ORF31 on the left-hand side of the genome based on the nomenclature system used for HVS (Fig 4). Restriction enzyme mapping and end-sequence analysis of two λDASH clones λVR3A and λVR4A covering the overlap between λD3-80 and λC7-1 revealed the presence of homologues of HVS ORF10 and ORF17 and permitted us to concentrate on the predicted DL-B region.

Initial DNA sequence analysis by the M13 dideoxy shotgun approach of a 2.5kb EcoRI subcloned plasmid from near the right hand side of λD3-80 revealed an ORF encoding a potential 337 amino acid TS protein with homology throughout its length to previously identified viral and cellular TS enzymes (Fig 5A). An amino acid alignment of the HHV-8 TS protein with those from HVS (12), H.ateles (HVA) (53), EHV-2 (61), varicella zoster virus (VZV) (24) and human DNA (59) revealed the closest relationship to the EHV-2 version (71% identity), slightly more divergence from the human and HVS TS proteins, and further divergence from the VZV TS protein (Table 1). Only the HVS and HVA versions are more closely related to each other.

Table 1. Comparison of the Overall Degree of Conservation between TS and DHFR of HHV-8 with Several Homologous Proteins

(A) Thymidylate Synthetase (TS)					
	HVS	HVA	EHV-2	VZV	HUMAN
HHV-8	66.4/79.5	65.4/78.2	71.6/82.7	61.8/78.1	67.3/79.2
HVS		84.4/93.7	66.1/80.3	66.0/79.6	70.4/83.3
HVA			67.1/79.9	63.2/70.3	68.7/84.7
EHV-2				65.1/81.0	73.4/83.7
VZV					68.7/84.7

(B) Dihydrofolate Reductase (DHFR)				
	HVS	HUMAN	PIG	DROSOPHILA
HHV-8	48.4/65.8	50.0/67.0	48.9/65.9	33.7/61.1
HVS		83.3/90.9	78.5/90.3	43.9/67.2
HUMAN			88.7/95.2	44.8/69.1
PIG				47.5/68.0

Values for percent identity (left) and percent similarity (right) of amino acid residues were derived from the output of GAP pairwise alignments with gap and length weights set at 3.0 and 0.1, respectively.

DNA sequence analysis of an adjacent 2.4kb EcoRI fragment subclone to the left of the TS gene within λD3-80 also revealed an HHV-8 gene encoding a complete 212 amino acid homologue of the HVS (62) and mammalian dihydrofolate reductase (DHFR) proteins (Fig 5B). However, the HHV-8 DHFR is highly diverged from both the H.saimiri and mammalian versions. For example, HVS DHFR is 83% identical at the amino acid level to

human DHFR and 78% identical to pig DHFR (59). In comparison, HHV-8 DHFR shows only 49% identity to the HVS, human and pig DHFR proteins, which is about an equivalent level of divergence as between the mammalian and Drosophila versions (Table 1). Furthermore, the residual homology to our original DHFR conserved primers proved to be far too low for the redundant PCR approach to have succeeded.

EXAMPLE 5

HHV-8 and HVS Encode Homologues of the BHV-4 IE1 Zinc Finger Protein:

An HHV-8 version of another recognizable gamma class herpesvirus gene was also found within the DNA sequence between the DHFR and TS genes. This ORF potentially encodes a 333 amino acid protein (Fig 6A) with a zinc-finger motif that resembles the prototype C3HC4 RING class zinc-finger motifs found in the herpes simplex virus IE110 (or ICP0) transactivator protein (27, 28, 51). However, the HHV-8 protein proved to be a variant of the newly recognized C_4HC_3 PHD/LAP class of zinc-finger motif (1, 55), which is most closely related to similar motifs in the IE1 protein of the bovine gamma-2 class herpesvirus BHV-4 (64). The BHV-4 major IE1 protein gene lies in the same orientation within the DL-B region of that virus and is encoded by a spliced four exon mRNA (64).

Subsequently, we also detected another upstream HHV-8 ORF mapping several kilobases to the right, but still within DL-B of λC7-1 that is predicted to encode a 256 amino acid protein (Fig 6A) with a second diverged copy of this same PHD/LAP subclass of zinc-finger motif. Further analysis revealed that BHV-4 also has a second duplicated but diverged copy of a PHD/LAP zinc finger motif in a potential downstream fifth exon of the IE1 gene, and that the positionally analogous 169 amino acid ORF12 protein of HVS also contains a closely related PHD/LAP class zinc finger motif. By analogy with BHV-4, we refer to these two HHV-8 ORFs as IE-1B and IE-1A. Both of the HHV-8 IE1-like genes contain intact ORFs with associated ATG

codons towards their 5'-ends, but the presence of excellent consensus splice acceptor motifs just upstream of their ATGs suggest that each may also be a component of a larger complex spliced transcript with upstream exons. A comparison alignment of the predicted IE-A and IE-B proteins revealed approximately 40% amino acid identity between the two (Fig 6A). Sequencing of PCR products obtained from HBL6, C282, 431KAP and AKS1 DNA confirmed the presence of virtually identical IE-1B regions in all five HHV-8 positive samples tested from both BCBL and KS sources.

Alignments of both PHD/LAP1 zinc-finger motifs from HHV-8 with the two BHV-4 IE1 and the HVS ORF12 motifs, and a comparison with six other known motifs of this type including one from *S. cerevesiae* yeast (SMM4) and three from C.elegans, together with those in the C7 protein in swine pox virus (40) and the ZYS-1A and ZYS-1B proteins of Chlamydomonas (63) are shown in Figure 7A. In all eleven of these proteins the LAP zinc finger motif is located close to the N-terminus, and we have tentatively placed them into a distinct subclass of LAP finger motifs with a central $CXCX_{4-7}H$ structure between C-3 and H-5 positions, compared to representatives of the majority of the PHD/LAP motifs, exemplified by HUMAF10 and HUMMLLa (55), which have $CX_{2-4}CX_4H$ in this region (Fig 7B). The RING zinc finger motifs of HSV ICP0, and the cellular RAG-1 protein and the PML and BRCA-1 proto-oncogene proteins are also shown for comparison (Fig 7C). All five of the gamma-2 herpesvirus IE1-like ORFs also contain at least three internal hydrophobic domains, suggesting that they may be membrane proteins rather than typical hydrophilic IE class nuclear transactivators.

EXAMPLE 6

A Fourth Potential Cytokine Gene and the T1.1 Nuclear RNA Also Map Within DL-B:

Virtually complete DNA sequence analysis of a 17-kb block encompassing the whole of the DL-B region of HHV-8 revealed fourteen

ORFs with clearly recognizable features. The flanking sections contained well-conserved homologues of HVS ORF10 and ORF11 on the left hand side and of ORF17, ORF18 and ORF19 on the right hand side. ORF6 to ORF9 and ORF17 to ORF19 are found at equivalent locations in all gamma herpesviruses, whereas ORF10 and ORF11 are present in HVS and in EHV-2, but are absent in EBV. As described elsewhere (21), a rightwards oriented but highly diverged HHV-8 homologue of HVS ORF16 proved to contain motifs characteristic of the human Bcl-2 family of anti-apoptosis proteins. Furthermore, despite showing only 15 to 20% overall amino acid identity to several cellular members of the Bcl-2 family, as well as to EBV BHRF1 and HVS ORF16, the HHV-8 homologue of Bcl-2 proved to be functionally active in blocking apoptosis induced by Sindbis virus infection (21).

Amongst the eight other recognizable homologues of cellular genes in HHV8 DL-B (six of which are either not encoded by or are not present at this location in the HVS genome), all mapped in the leftward orientation between ORF11 and ORF16 (Bcl-2), in the following order from left to right: vIL6, DHFR, IE-1B, TS, vMIP-1B, BCK (β-chemokine-like), IE-1A and vMIP-1A (Fig 4). Identification of the vIL-6, vMIP-1A and vMIP-1B genes, which potentially encode proteins with between 25 and 40% identity to human IL-6 and MIP-1α or MIP-1β respectively, and evidence for functional activity of vIL-6 has been presented elsewhere (50). The predicted BCK-like protein of 114 amino acids (Fig 6B) represents a third potential HHV-8 encoded β-chemokine with a fairly typical $CCX_{15}CX_{22}C$ cysteine bridge pattern (8) and a probable hydrophobic N-terminal signal peptide region, but unlike the two vMIP-1 proteins, it shows only low level homology to several of the closest known cellular members of this family (such as MIP-1α, RANTES, TAC-2, MCP-1, and CC; Fig 6B). Conservation of the cysteine bridge pattern between HHV-8 encoded vIL-6 and human IL-6 is also illustrated in Fig 6C. The 2.7-kb divergent region between vMIP-1A and Bcl-2 (Fig 4) appears to contain multiple transcriptional control elements, which are in the process of being

characterized (Chiou, C.-J. and Hayward, G.S., unpublished data), as well as a rightwards-oriented gene lying just upstream from the Bcl-2 homologue that encodes the abundant 1.1-kb lytic cycle nuclear RNA (T1.1) described by Zhong et al (69). Finally, a central approximately 3.4-kb region between BCK and IE-1A contains another complex region with multiple binding motifs and two sets of 20-bp and 30-bp high (G+C) repeats totalling up to 630-bp that tend to delete in λ clones (denoted by Rpt in Fig 4).

EXAMPLE 7

Identification and Expression of mRNAs in Butyrate Induced BCBL Cells:

Although KS-derived spindle cell lines do not contain HHV-8 DNA (38), several cell lines from BCBL tumors that do stably maintain both HHV-8 and EBV DNA have been described (16, 17, 45). We have used the HBL6 subline of BC-1 cells to examine whether or not transcripts for some of the genes described here could be detected. Anti-sense riboprobes for IE-1A, IE-1B, vMIP-1B and DHFR were generated and used for Northern blot hybridization with total cell RNA prepared from HBL6 cells both before and after attempts to induce the lytic cycle with agents that are effective with some EBV cell lines. Initial experiments revealed that an abundant IE-1A mRNA species of 1.2-kb, and a less intense IE-1B mRNA species of 2.0-kb were expressed after treatment with butyrate, but not with TPA or 5-azacytidine and not in untreated cells (Fig 8A). Others have also reported that TPA fails to induce HHV-8 lytic cycle expression in BC-1 cells, but that butyrate does do so (44, 45). We also found a single transcript of 0.8-kb for MIP-1B in HBL6 cells at 12 h after butyrate induction, but not in uninduced cells. Both the IE-1A and vMIP-1B mRNAs were absent at 4-h after butyrate treatment and their synthesis was blocked in the presence of cycloheximide for either 12 or 24 h (Fig 8B, lanes 3 and 6). Single transcripts of 1.1-kb corresponding to HHV-8 DHFR mRNA (Fig 9B) and of 1.0-kb corresponding to vIL-6 mRNA (50)

were also detected after butyrate induction. A time course experiment showed that neither the IE-1A nor DHFR transcript was detectable at 4 or 8 h after butyrate addition, but that they both peaked at 12 h and declined considerably by 24 h and 48 h after induction (Figs 9A and 9B). Again, addition of cycloheximide also completely blocked expression of DHFR mRNA in 12h butyrate-induced cells (not shown). Exactly analogous results have also been obtained with RNA after butyrate induction of the HHV-8 positive, but EBV negative, BCBL-1 cell line (Fig 9C). Therefore, each of the genes tested gives rise to a distinct transcript and they all appear to represent primarily lytic cycle rather than latently expressed genes.

Northern Blot mRNA Analysis:

The HBL6 subclone of BC-1 cells derived from a human BCBL/PEL tumor containing both HHV-8 and EBV genomes (16) was grown in RPMI medium plus 20% fetal calf serum. Induction treatments involved addition of either 4mM sodium butyrate or 5μM 5-azacytidine at the 0 h time point. In some cultures, cycloheximide was added at 50μg/ml at the time of induction to block protein synthesis. Cells were harvested at various time points and total cell RNA was extracted with Trizol (BRL, Bethesda). RNA samples (5 μg/well) were subjected to electrophoresis on 2% formaldehyde/0.9% agarose gels in MOPS buffer (20mM MOPS, pH7.0, 10mM NaOAc, 2mM EDTA) and transferred to nylon membranes (S and S Nytran) by capillary transfer. Hybridization was carried out at 68° overnight in 6x SSC, Denhardts and 0.1% SDS with 10^6 cts/min/ml of [^{32}P]-labeled riboprobes generated by in vitro synthesis with T7 or SP6 RNA polymerase from appropriate plasmid DNA templates. All four intact ORF riboprobe template plasmids represented PCR DNA products derived from λD3-80 or λC7-1 subcloned into the pGEM (IE-1A and IE-1B, SP6), Bluescript KS (vMIP-1B, T7) or pSG5 (DHFR, T7) vectors.

EXAMPLE 8

Biological Activity of vIL-6

To examine whether vIL-6 specifies a functional gene product with properties analogous to those of human IL-6, we utilized the murine hybridoma cell line B9 that has been shown previously to be dependent on IL-6 for growth. Both murine and human IL-6 are effective in supporting B9 growth. Rat embryo fibroblast (REF) cells were transfected with expression plasmids containing vIL-6 ORF sequences in the positive (pvIL-6) or negative (pvIL-6neg) orientations relative to the human cytomegalovirus promoter-enhancer, and supernatants were harvested between 2 and 4 days posttransfection. Various dilutions of the extracts were made and assayed for IL-6 activity relative to recombinant human IL-6 (rhIL-6). The results shown in Fig. *10* indicate that the vIL-6 gene product is capable of supporting the growth of B9 cells and therefore that it displays functional activity analogous to human and murine IL-6. The receptor for human IL-6 has been identified as an 80kD protein (IL-6R) that, when bound to IL-6, associates with a transmembrane protein, gp130, thought to represent the signal transducer. Utilizing a functionally neutralizing monoclonal antibody directed against the murine IL-6R (D7715A7, Pharmigen), we repeated the B9 assays in the presence or absence of the antibody to determine if IL-6R played a role in mediating the observed vIL-6 activity. The results (Table 2) show that the anti-IL-6R antibody inhibited both vIL-6 and rhIL-6 activity in the B9 assay, indicating that this function is mediated through the same signal transduction pathway utilized by endogenous IL-6. A monoclonal antibody directed against murine IL-2 (S4B6, Pharmigen) had no effect on vIL-6 function in parallel assays (Table 2).

To demonstrate further the functional equivalence of vIL-6 to endogenous IL-6, we also examined the ability of vIL-6 to induce the expression of a representative acute-phase gene, α_1-acid glycoprotein (AGP), in the hepatoma cell line Hep3B. Human IL-6 is known to mediate the induction of acute-phase mRNA and protein synthesis in hepatocytes, and this effect is mediated in part through the activation of a nuclear factor that binds

to common promoter elements of these genes. To determine whether vIL-6 also displays this property, we transfected Hep3B cells with either of two vIL-6 expression vectors (pvIL-6 or pSVvIL-6), or with appropriate negative control plasmids, and harvested the cells for RNA 48h posttransfection. RNA was also harvested from untreated or rhIL6-treated Hep3B cells for comparison. Northern blot hybridization analysis was undertaken to determine the relative levels of AGP mRNA present in the cells (Fig.10). The results revealed that AGP mRNA levels were induced in response to vIL-6 as well as by rhIL-6, demonstrating the functional equivalence of the viral and endogenous human IL-6 cytokines for this activity.

We have demonstrated here that HHV-8 encoded vIL-6 is functionally equivalent to human IL-6 in supporting B9 cell growth and activating acute-phase gene expression *in vitro*. It is also possible that vIL-6 specifies other, *in vivo* functions analogous to endogenous IL-6, and that it could be involved in KS, Castleman's disease, and other malignancies. The potential role of vIL-6 in KS in particular, could in principle be mediated through expression of this protein during either latent or lytic infection in affected tissues.

vMIP-1 (A and/or B) could also play a role in HHV-8-associated disease, perhaps cooperatively with hIL-6/vIL-6, by effecting local increases in cytokine levels by recruitment of cytokine-producing cells into infected tissues. It can also be postulated that the vMIP-1 proteins may have a more direct role in effecting cellular proliferation by acting as mitogens, since MIP-1α transcripts have been detected at high levels in human leukemic cells, suggesting a potential involvement of this cytokine in neoplastic transformation. Therefore, the presence of vIL-6 and vMIP-1 genes in HHV-8 may be directly relevant to cellular transformation and the proliferation characteristics of the cells with which the virus is associated. Furthermore, since human MIP-1α, MIP-1β and RANTES all compete with HIV gp120 for binding to T-cells, and each can both block HIV entry into T-cells as well as stimulating HIV growth in macrophages, there appears to be great potential

here for reciprocal interactions between HIV and HHV-8 that could influence the progression of AIDS in patients infected with both viruses.

Cloning and Expression of vIL-6. The coding region of vIL-6 was obtained by PCR amplification of appropriate sequences from the lambda phage clone D3-80 using oligonucleotide primers directed to the 5' and 3' ends of the vIL-6 ORF and containing *Bam*HI sites within 5' non-complementary sequences. The vIL-6 ORF was cloned as a *Bam*HI fragment into the *Bam*HI site of the eukaryotic expression vectors pcDNA.3 and pSG5 (Stratagene, Inc.). Plasmids containing vIL-6 coding sequences in the correct (pvIL-6) and negative (pvIL-6neg) orientations relative to the HCMV promoter-enhancer and 3' processing/polyadenylation signals in pcDNA.3 were obtained. The plasmid pSVvIL-6 contains the vIL-6 ORF expressed under the control of the SV40 promoter in the vector pSG5. Rat embryo fibroblast cells (REF) were grown in MEM containing 10% fetal calf serum (FCS), and transfected with 5μg of either pvIL-6 or pvIL-6neg by calcium phosphate coprecipitation. Supernatants were harvested 2 to 4 days posttransfection for IL-6 functional assays using IL-6 dependent B9 cells. Hep3B cells grown in MEM/10% FCS and were transfected with 10μg of pvIL-6, pvIL-6neg, pSVvIL-6, or pSG5 and harvested 48h posttransfection in assays to measure AGP induction as a function of vIL-6.

Assay for IL-6 Function. The IL-6 dependent murine cell line B94 was obtained from L. Aarden and was maintained in RPMI supplemented with 10% fetal calf serum and 100U/ml IL-6. Supernatants from vIL-6-transfected cultures were diluted in RPMI/10%FCS and 100μl containing $2x10^5$ B9 cells in log phase growth added in quadruplicate to 96 well plates. After 72h at 37°C, 20μl of 5mg/ml MTT was added to each well and the cultures incubated for a further 4h. 75ul of a solution containing 20% SDS, 50% DMF (pH4.7) was then added to each well and the plates incubated in the dark overnight at room temperature to dissolve the crystals. $O.D._{570}$ readings were taken. Values in units were obtained by selecting dilutions of culture supernatants

giving linear results and interpolating on a linear portion of a standard curve. Assays in which antisera to IL-6R or gp130 were used were carried out similarly and were performed in duplicate. Rat monoclonal antibodies to murine IL-6R (IgG_{2b}) and murine IL-2 (IgG_{2a}) were obtained from Pharmingen (cat# 18400D and 18000D, respectively).

Table 2. Inhibition of vIL-6 activity by anti-IL-6R monoclonal antibody.

(a) hIL-6

10pg/ml hIL-6	Absorbance$_{570}$	% Inhi-bition	10pg/ml hIL-6	Absorbance$_{570}$	% Inhi-bition
0.0μg/ml αIL6R	0.453 +/- 0.034	-	0.0μg/ml αIL2	0.453 +/- 0.034	-
2.5μg/ml αIL6R	0.182 +/- 0.023	59.2	2.5μg/ml αIL2	0.371 +/- 0.011	17.5
5.0μg/ml αIL6R	0.139 +/- 0.021	68.7	5.0μg/ml αIL2	0.371 +/- 0.022	17.3
10.0μg/ml αIL6R	0.089 +/- 0.014	80.0	10.0μg/ml αIL2	0.342 +/- 0.043	23.4

(b) vIL-6

1:40 vIL-6 sup	Absorbance$_{570}$	% Inhi-bition	1:40 vIL-6 sup	Absorbance$_{570}$	% Inhi-bition
0.0μg/ml αIL6R	0.420 +/- 0.030	-	0.0μg/ml αIL2	0.420 +/- 0.030	-
2.5μg/ml αIL6R	0.277 +/- 0.005	33.7	2.5μg/ml αIL2	0.380 +/- 0.016	9.5
5.0μg/ml αIL6R	0.229 +/- 0.003	45.2	5.0μg/ml αIL2	0.398 +/- 0.022	8.2
10.0μg/ml αIL6R	0.193 +/- 0.001	53.8	10.0μg/ml αIL2	0.364 +/- 0.009	12.8

B9 cell proliferation assays were carried out essentially as described in the legend to Fig.*10* either in the absence or presence of added monoclonal

B9 cell proliferation assays were carried out essentially as described in the legend to Fig.*10* either in the absence or presence of added monoclonal antibody (2.5 to 10.0μg/ml) to the murine IL-6 receptor or murine IL-2 (negative control). Data are derived from MTT assays performed in triplicate for each sample and are presented with B9 proliferation set at 100% in the absence of antibody and 0% is the value obtained when medium lacked the addition of reference IL-6 (rhIL-6) or transfected cell supernatant (vIL-6). Activities of rhIL-6 and vIL-6 used in these assays were comparable to those obtained previously (Fig. 10).

EXAMPLE 9

Overexpression of KSbcl-2 inhibits Sindbis virus-induced apoptosis. Sindbis virus, an alphavirus, has been shown to induce typical apoptosis . We have established a Sindbis virus vector system to test the ability of candidate death-regulatory genes to delay or accelerate Sindbis virus-induced apoptosis. The putative KSbcl-2 coding sequence with a N-terminal HA-tag was cloned into the Sindbis virus vector. KSbcl-2 provided to be equivalent to Bcl-2, Bcl-x_L and BHRF1 in its ability to inhibit Sindbis virus-induced apoptosis as indicated by approximately 50% cell viabilities at 48 hours post-infection compared to 2-3% with an irrelevant gene (CAT) or with KSbcl-2 in the reverse orientation. We and other have shown that the HA-tag has no effect on the anti-apoptotic activity of Bcl-2 homologs. To verify the expression of KSbcl-2 protein, infected cell lysates were analyzed by immunoblotting with anti-HA antibodies. A protein of 24 kD was observed only in cells infected with the recombinant virus encoding KSbcl-2. To assess the role of the BH3 domain in the death-suppressor activity of Bcl-x_L, amino acid substitutions were introduced into the Bh3 domain of Bcl-x_L (Glu to Gin change at residue 92, Asp to Ala change at residue 95 and Glu to Asp change at residue 96) and the anti-death function was assayed using the Sindbis virus vector system. The BH3 mutant was as efficient as wild-type Bcl-x_L at inhibiting apoptosis,

indicating that BH3 may not be directly involved in the death-suppressor activity of Bcl-x_L.

Production of recombinant Sindbis virus. dsSV vectors containing KSbcl-2 in both orientations, chloramphenicol acetyltransferase (CAT), bcl-2, bcl-x_L, BHRF1, or the BH3 mutant of bcl-x_L were each linearized with Xho I and *in vitro* transcribed using SP6 RNA polymerase. Stocks of recombinant viruses were generated by transfecting the infectious RNA into BHK (baby hamster kidney) cells and collecting the supernatant at 24h post transfection. Virus titers were determined by standard plaque assays.

Cell viability and immunoblotting. BHK cells were infected with different recombinant dsSV viruses at a multiplicity of infection (MOI) of 5 plaque forming units per cell, and cell viability was determined by the trypan blue exclusion method at 48h post-infection. At 16h post-infection cell lysates were prepared from BHK cells infected with recombinant viruses at a MOI of 5 and were immunoblotted with 12CA5 anti-HA antibody (Berkeley) and detected by ECL (Amersham).

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